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Crude Oil Metagenomics for Better Bioremediation of Contaminated Environments

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1. Introduction

Our planet suffers more and more from various pollution problems. The marine environments are always regarded as sewers without end and are then subjected to different types of toxic rich rejects leading to oceans and seas degradation. The marine environment becomes at the same time the witness and the actor of the history of the planet, and its chemical composition witnesses all the complexity of its evolution. Coastal regions are often where we find various pollutants. Seawater (Jaffrennou et al. 2007; Pérez-Carrera et al. 2007), marine sediments (Wakeham, 1996), and interstitial water (Pérez-Carrera et al., 2007) have shown this pollution. Petroleum hydrocarbons are among the most toxic compounds poured at sea. Known as the most significant pollutant, crude oil can persist for years (Burns & Teal 1979), with dangerous effects on coastal environments and negative effects on both the ecosystem and the marine biodiversity (Clark 1992; Rice et al., 1996). In marine environment, crude oil is subjected to physico-chemical and biological modifications, which enhance hydrocarbons solubility in the water and consequentially cause extensive damage to marine life, natural resources, and human health.

It is estimated that most petroleum compounds have carcinogenic properties (Ericson et al. 1998; Aas et al., 2000; Shaw & Connell 2001). Because of their toxicity, several hydrocarbons are classified by the Agency of Environmental Protection, the World Health Organization, and the European Union as top priority pollutants. Based on their distribution and their high toxicity, hydrocarbons are considered as the principal organic markers of the anthropogenic activity in the ecosystems (Laflamme & Hites, 1978; Bouloubassi & Saliot, 1991; Budzinski et al., 1997; Yunker et al., 1996; Fernandes et al., 1997).

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Crude oil is composed of hundreds of compounds (Bertrand & Mille 1989). The major fractions are the non-aromatic hydrocarbons (NAH) and the aromatic hydrocarbons (AH). The NAH fraction includes aliphatic hydrocarbons, branched isoalkanes such as pristane and phytane, and cycloalkanes which are represented by an unresolved complex mixture (UCM). Of great interest to several research groups are non-aromatic and aromatic oil hydrocarbon fractions, such as the contents of total aliphatic (TA), total resolved or n-alkanes, total aromatics, and polyaromatic hydrocarbons (PAHs). n-Alkanes represent a group of non-polar and photocatalytically stable organic compounds. Their characteristics provide useful information on the origin of biological and/or petrogenic sources of pollution. At the same time, aromatic hydrocarbons, and particularly PAHs, represent a group whose characteristics and individual proportions are useful in comparative studies of petrogenic and pyrolytic sources (Clark 1992; González et al., 2006; Rice et al., 1996). Characterization of hydrocarbons and their specific sources are important for understanding the crude oil fate and behaviour in marine environment and for predicting their potential long-term impact, which allow taking effective clean-up measures in the specific marine compartment. Therefore, there is an increasing need to develop easy and accurate methods for removing hydrocarbon from seawater in the case of an oil spill. The removal of petroleum hydrocarbon or its transformation into less toxic products by bioremediation is a less invasive and less expensive process if compared to classical decontamination. However, the use and optimization of bioremediation treatments in the water compartment require knowledge of the seawater microbial communities directly and indirectly involved in the degradation of hydrocarbons.

Multiple initiatives have been developed to resolve the problem of petroleum pollution. An array of procedures has been developed including physical, chemical, and biological techniques. Among these procedures, bioremediation is currently used alone or associated to physicochemical procedures. Biological methods of rehabilitation of polluted sites represent an interesting alternative. These techniques are based on the microorganism's capacities to degrade petroleum compounds (Harayama et al., 1999). Indeed, within the last two decades, the use of molecular techniques has led to a significant improvement in our knowledge of microbial diversity in different complex environments. Hence, it becomes necessary to characterize microbial communities in polluted environments; especially when the pollutant is as complex as crude oil. Thus, characterization of the microbial diversity and the identification of microbial key players in the degradation of crude oil could be useful in defining new strategies for bioremediation (Prince et al., 1993).

Prokaryotes have been extensively documented in hydrocarbon-contaminated environments (sediment, seawater, soil, activated sludge, ice, estuary and river) as well as during oil degradation. Comparisons of the microorganism's phylogenetic diversity from multiple petroleum contaminated environments are of great importance. This information allows improving our knowledge on the active core members of this group and to understand whether the composition of this bacterial group changes in response to specific petroleum substrate and environmental parameters. The ability to understand such changes and to correlate them to microbial activities through population and phylogenetic inventories is especially important to understand naturally dynamic environmental parameters. This will help in setting up strategies for optimizing environmental conditions that could improve the hydrocarbon biodegrader's effectiveness towards bioremediation.

In fact, to combat petroleum pollution and for an effective intervention in case of accidental or chronic discharge, the study of the bioremediation process is necessary. Bioremediation rests on the use of the natural capacities of certain organisms to metabolize pollutants (Serrano et al., 2007). It is necessary to improve our knowledge on the microbial communities involved in the metabolisation of hydrocarbons and to follow their process dynamics to optimize the bioremediation techniques. However, in the majority of the natural ecosystems, the study of the microbial communities, their densities, and their diversity is difficult (El Fantroussi et al., 2003; Curtis & Sloan, 2004, Elloumi et al., 2008). Because microorganisms are not easy to isolate due their diversity, their organization in consortia, their dynamic and specific cultivation characters, molecular techniques must be used to identify the effective players (microorganisms) in bioremediation. These techniques promote the identification, phylogenetic diversity analyses, and the study of the metabolic diversity of the microbial communities present in petroleum contaminated sites. Currently, several researches aim to establishing correlations between the microbial diversity, the nature, and levels of hydrocarbon in the polluted environment (Head et al., 2006). Thus, the molecular approach could allow the establishment of inventories of the microorganism's composition in polluted environments and to elucidate the "black box" of this composition in terms of abundance and thereafter in terms of adaptation and functionalities.

2. Crude oil as an important source of energy in the world

2.1 World production of oil and its derivatives

One hundred years ago oil exploitation began, first as a source of energy and later to include oil as a source of raw material. As a source of energy, crude oil went through an uninterrupted progression of its extraction during more than one century, driven by development of transport and industry. The annual report of the OPEC in 2008 showed an increase in the oil production worldwidwe. Indeed, the annual production of oil products was about 11 million tons per day (11 Mt/d) in year 2000 with an increase in the production of 1.9%/year in the current decade. Estimates of the worldwide oil consumption, suggest an increase of about 44.7 million barrels per day (that is to say 6.4 Mt/day) between 1999 and 2020, which corresponds to an increase in oil consumption from 74.9 Mb/d (either 10.7 Mt/day) in 1999 to 119.6 Mb/d (or 17 Mt/day) in 2020. The annual growth is thus estimated at 2.3 % /year, whereas it was only of 1.6 %/year between 1970 and 1999. Fig.1 shows the distribution of the worldwide total oil (Gasoline/Naphtha, Fuel oil, Middle distillates and others product) production. It is estimated that one trillion barrels have already been harvested, and about 3 trillion barrels of oil remain to be recovered worldwide. Oil production is expected to peak sometime between 2010 and 2020, and then fall inexorably until the end of this century.

2.2 Sectors of use

Fig. 2 presents the worldwide consumption of petroleum during the 20th and 21st centuries. The increase in the world production of oil is in direct relationship to the world requirement of oils and its derivatives. This need is imposed by various sectors (Fig. 2), for instance, the transportation sector is the greatest consumer of petroleum products, and its avidity for petroleum will increase until year 2030. The other sectors are mainly: industry, electricity

generation, agriculture, and marine activities that include marine transportation, off shore oil production and fishing, among others.

Our society faces the challenge of increasing the production of goods and services for a growing population using new process technology that should be energetically efficient and environmentaly friendly. This also will be the case for the petroleum industry. The primary target of the petroleum industry is to enhance and maintain a continuous oil production.

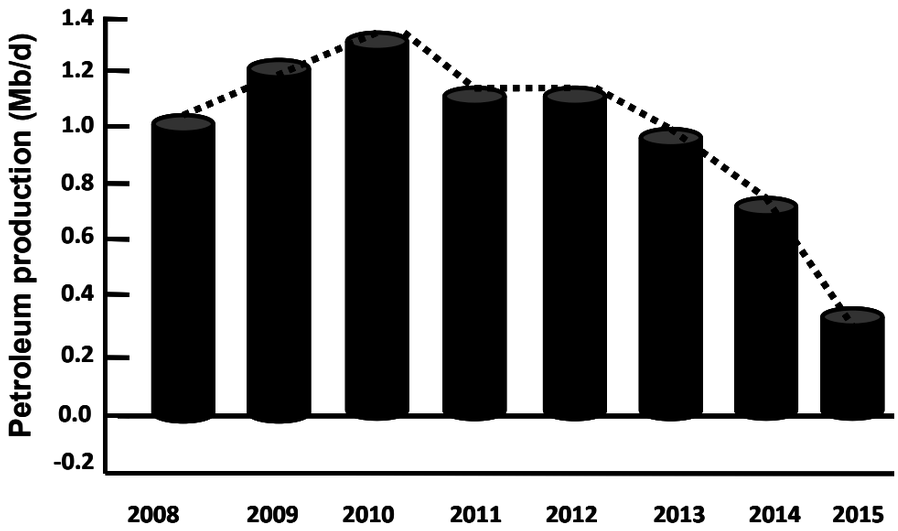

Fig. 1.Estimation of petroleum production worldwide. Adapted from OPEC (2008) with modifications.

3. Crude oil as a principal source of pollution in the world

The problems involved in the excessive production and consumption of oil are increasingly numerous and at various levels, which is directly related to the impact of industrialized factories on the environment (Killops & Killops, 2005).

3.1 Various aspects of marine oil pollution

Oil is a great source of pollution to the marine environments in the world, which can largely influence the ecological balances and the economic activities in the areas of interest. The National Research Council estimated in the review "Oil in the Sea: inputs, fates and effects, 2002 ", that the quantity of oil introduced annually into the oceans through different ways is 1.3 million tonnes/year. Take into consideration that one ton of oil can cover approximately a surface of 12 Km^2.

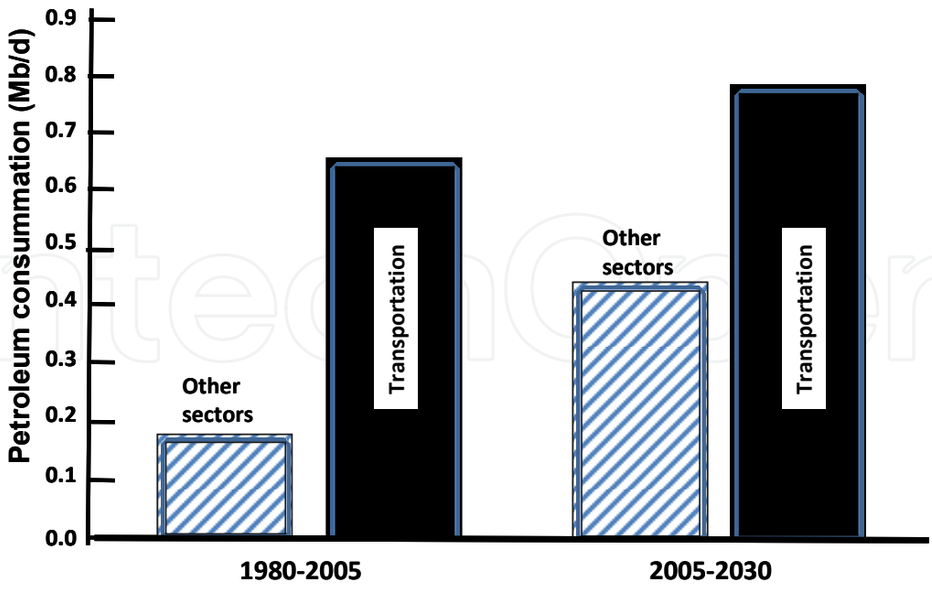

Fig. 2. Estimation of the worldwide consumption of petroleum products. Adapted from OPEC (2008), with modifications.

Oils are generally rejected close to the refineries or in the ports (telluric pollution), but also in open sea by the discharge of water from tankers ballast (approximately two million tons of hydrocarbons can be rejected by deballasting each year) or accidentally by spillage (pelagic pollution) (Marteil, 1974). Oil spills in the sea due to accidents, represent a fewer proportion of the total hydrocarbons poured in seawater each year. An imperceptible but daily source of oil discharge in the sea is represented by wastewater discharges (industrial and domestic wastes). The total hydrocarbon content in wastewater ranges from 200 to 1800 mg/day/habitant (Louati et al., 2001). Multiple examples in the world confirmed that the most significant catastrophes are related to oil pollution such as those recorded in 1967 (Spain, France, USA) and in 2010 (Gulf of Mexico, USABP platform). This catastrophe refers to the April 20, 2010 explosion and subsequent fire on the Deepwater Horizon semi-submersible Mobile Offshore Drilling Unit (MODU), which was owned and operated by Transocean and drilling for BP in the Macondo Prospect oil field about 40 miles (60 km) southeast of the Louisiana coast, in this catastrophe a total of 4.9 million barrels of crude oil were released into the Deepwater Horizon, making this the biggest oil spill to have occurred in US-controlled waters (Jarvis, 2010) (Source: Alice-Azania Jarvis http://www.independent.co.uk/environment/bp-oil-spill-disaster-by-numbers-2078396.html). War is also a significant source of pollution. Indeed, the Gulf war in the Middle East, in 1991 the destruction of crude oil field's and spills from oil tankers that were bombarded caused the pouring of significant quantities of crude oil into the marine environment (Bingham, 1992). The total assessment was estimated at 800 000 tons poured, 40 million tons of water-logged soils with oil, and 700 km of polluted coasts. It was estimated that since 1960 500 million of gallons of oil were poured by oil tankers in the European seas and the Pacific. In China, pollution by hydrocarbons is primarily in lakes and rivers as a result of the industrialization increase.

3.2 Fate of crude oil in the marine environment

The distribution of hydrocarbon pollutants in the environment comes from different sources. Once the oil spill takes place and in spite of their relative chemical stability and their persistent character, hydrocarbon pollutants are carried from an environmental compartment to another by undergoing in each one of them specific transformations such as physical, chemical, and biological modifications (Fig. 3).

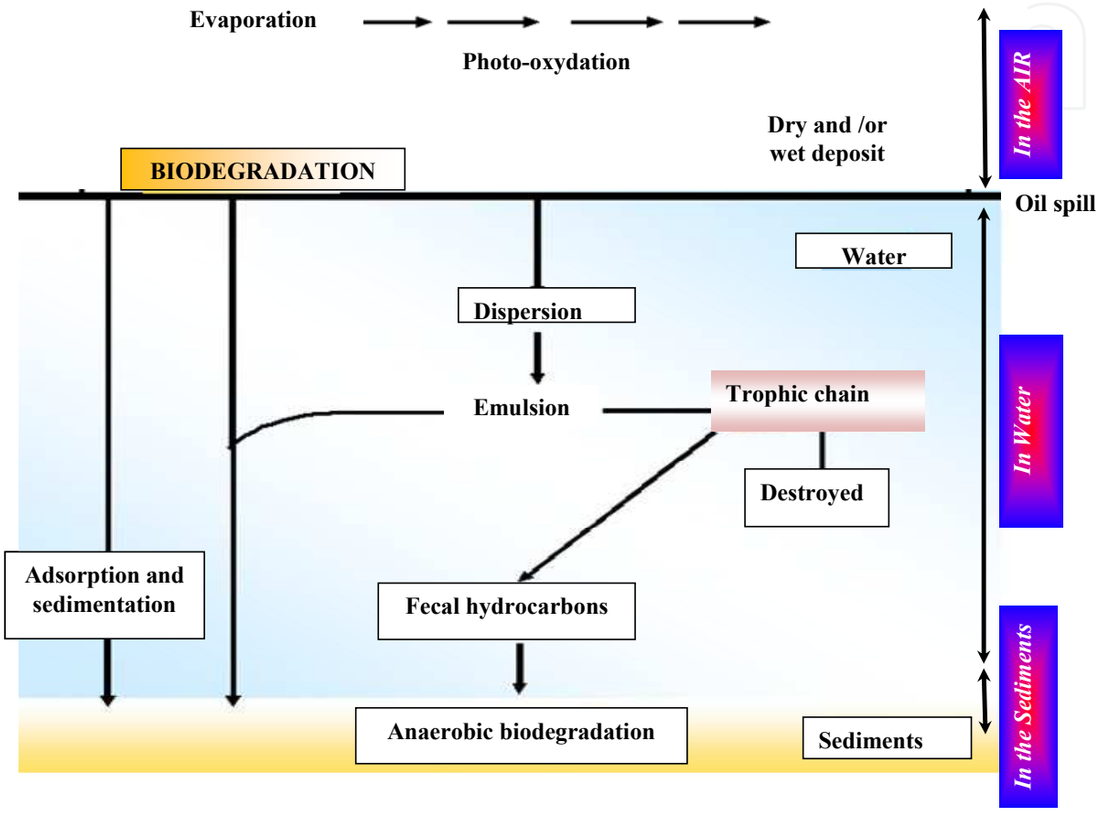

Fig. 3. Physico-chemical and biological parameters involved in marine environment after a crude oil spill (Bertrand & Mille, 1989, with modifications).

3.2.1 In the air

Atmospheric rejections of hydrocarbons have as the principal source the anthropic activity and specifically the heating by fuel, coal, and wood combustion. Indeed road transport (black fume from diesel engines); oil industries (refining, catalytic cracking of oil), production of aluminum or iron; treatment of wood by creosote; the asphalt of roads, and the cigarette smoke represent important sources of this anthropic activity. The contribution of the biogenic sources in the atmospheric contamination is supported by volcanic activities, decomposition of organic matter and the control of fires (drill, agriculture). In the air, hydrocarbons are distributed between the gas and particulate phases (volatile, semi-volatile or particulate) according to their vapor pressures, their molecular weights, and the ambient temperature (Atkinson & Arey, 1994; Oda et al., 1998). The transportation of hydrocarbons by air masses can be done locally or on a large scale. In fact, the presence of hydrocarbons

has been detected at several hundreds of kilometres or even several thousands of kilometres from their emission source (Ramade 1992; Bouchez et al., 1996). Hydrocarbon carrying into the air depends mainly on the intensity of the emission, its altitude, the size, and the chemical stability of the molecules concerned (Ramade, 1992). The presence of hydrocarbons in the air represents a significant source of contamination for the marine environments due to the phenomenon of deposit.

3.2.2 In water

Significant discharges of petroleum products occur in continental and littoral water (oceans). According to Palayer (1997) the origin of water pollution by organic compounds derives from seven sources, which include:

- Runoff waters, which mainly contaminate the surface water
- Domestic and industrial effluents rejected after passage through wastewater stations
- Industrial effluents (washings of produced gases, coals cooling, etc.)
- Spent oils such as oil draining following rejection
- Dry and wet deposits coming from the atmosphere
- Water pipelines with an interior coat of tar that could salt out hydrocarbons in distributed water as the pipelines age.
- Accidents related to oil activities during oil extraction, refining, and oil spills.
- Natural oil escapes

To these sources cited by Palayer, is convenient to add water drainage and the scrubbing of the agricultural grounds contaminated with hydrocarbon products (McKiney et al., 1999). It is also possible to include as contamination sources other discharges related to the oil activity specifically the degasification of oil tankers. The multiplicity of the sources of water pollution by hydrocarbons makes very difficult the identification of the main sources of contamination.

The distribution and concentration of hydrocarbons in contaminated waters are influenced by several parameters such as the water pH, the salinity, and content of organic matter in suspension (Fernandes et al., 1997). In general, hydrocarbon concentration in bulk water is relatively low compared to hydrocarbon concentrations in sediments (Zrafi-Nouira et al., 2008; Zrafi-Nouira et al., 2009; Zrafi-Nouira et al., 2010) owing to the fact that hydrocarbon contaminants are absorbed easily on the organic matter in particulate and colloidal form (Baumard et al., 1998a). In the aqueous compartment, hydrocarbons are subject to degradation similar to the hydrocarbon degradation observed in the atmosphere. For some hydrocarbon compounds, volatilization ensures the transfer of hydrocarbons from the water surface towards the atmosphere as is the case of low-weight molecular compounds. While high-molecular weight compounds tends to precipitate and settle on the sediments where they accumulate. Finally in water, the oil accumulation by the living organisms also contributes to the reduction in the concentration of hydrocarbons (Baumard et al., 1998b).

3.2.3 In the sediments

Hydrocarbon concentration in sediments is in general relatively higher than hydrocarbon concentration in air and water, however the concentration of hydrocarbon in sediments are in direct relation with its concentration in the watery and atmospheric compartments (Gao

et al., 1998). Simpson et al (1998) stated that hydrocarbon contamination in sediments and its compositions are related to its origin. However, many environmental processes contribute to skew this "signature" while acting selectively on each compound as in the case of degradation by microorganisms. In addition, the distribution of hydrocarbons, in the sediments, between the aqueous phase and the solid phase depends on the coefficient of division n-octanol/water (log Kow) of each compound as well as the nature of the sediments (content of clay, silts, and carbon) (Palayer, 1997). After adsorption on the sedimentary particles, hydrocarbon is accumulated, and reach concentrations higher than the concentrations of hydrocarbon found in the water column, even though contaminant concentration decreases as the distance from the source increases following a logarithmic trend (Tuvikene, 1995). The adsorption of hydrocarbons in the sediments makes them more stable and less accessible to biological degradation. Thus, these pollutants can persist in the sediments several months even several years. That explains why sediments are considered tanks of pollutants (Ollivon et al., 1993; Djomo et al., 1995; Baumard et al., 1998). There are two phases of hydrocarbon accumulation in the sediments:

- A reversible phase (unstable) in which there is attachment of hydrocarbons on the sediments surface. This phase reflects the biodisponibility of hydrocarbons for delivery in the aqueous circulation compartment by bioturbation (agitation of the sediments by invertebrates) and suspension or diffusion. Thus, in this phase the biogeochemical cycle of these compounds could be initiated (Ramade, 1992).
- An irreversible phase (stable) which is a slow process that corresponds to the diffusion of the contaminant towards the sedimentary organic fraction.

Thus, the accumulation of hydrocarbons in the sediments is not definitive. The delivery of sedimentary hydrocarbons in solution or suspension can have harmful effects on the watery organizations (Beckles et al., 1998). However, only a small fraction of sedimentary hydrocarbons is subject to dissolution. Hydrocarbons are less persistent in the aerobic surface zone of the sediments than in the anaerobic deeper layers (Wilcok et al., 1996). The examination of the spatial distribution of hydrocarbons in the sediments is a determining factor in the evaluation of the biodisponibility of these compounds for the watery organizations and the identification of the possible sources of contamination (Gao et al., 1998). However, it has been shown that variation of hydrocarbons concentration in the sedimentary compartment does not reflect necessarily a pollution variation, but rather a change of the properties in the hydrocarbon/sediment interaction (Schilderman et al., 1999).

4. Bioremediation of hydrocarbon pollution in marine environment

It is clear from the description of the hydrocarbons fate in the different marine compartments that petroleum compounds are subjected to natural degradation by the native microorganisms. This degradation limits the dispersion of petroleum hydrocarbon in water and minimizes the pollution levels in some degree. However, natural degradation of hydrocarbons is not sufficient and can not eliminate the bulk of the pollution fraction in the environment. Thus, the application of biotechnology in the elimination of crude oil pollution is necessary. In fact, environmentally-related biotechnological processes were pioneered in the petroleum industry. Oil spill bioremediation technologies use modern environmental techniques that are based on natural processes to remove spilled oil from the environment without undesirable environmental impacts.

4.1 Bioremediation

Bioremediation regroup strategies that allow microorganisms the elimination of oil pollution. In fact, few pollutants resist indefinitely the attack of natural microbiota. However, the time required for the natural degradation of pollutants is extremely variable and can reach several decades. This explains the necessity of human intervention to accelerate this process. Biological methods are often cheaper than other types of treatments (Van Hamme et al., 2003). Microorganisms, which are present in natural environments, have a significant potential for the biological degradation of hydrocarbons. Bioremediation is a process that can be adopted *in situ* or *ex situ*. It's a process that uses indigenous microorganisms in recycling specific pollutants through a series of complex chemical reactions. Metabolized organic waste is transformed into water, carbon dioxide, and other sub-products that provide bacteria the energy needed for their development (Prince, 1993; Shwannell et al., 1996). The result of a total biological degradation is the elimination of pollutants without producing toxic or dangerous residue. The de-pollution by biological means attempts to accelerate or stimulate the processes of biological degradation in order to reduce the contents of contaminants. Though this approach remains a subject of controversy, several bioremediation processes have been carried out worldwide following accidental oil discharges. Romantschuk et al. (2000) showed the effectiveness and the reproducibility of the *in situ* bioremediation of a ground contaminated by crude oil. Margesin & Schinner (2001) confirmed the effectiveness of the bioremediation on site. In fact, they applied an in-situ bioremediation for the decontamination of a ground polluted by crude oil in the Alpine Glacier area.

Bioremediation represents an interesting alternative to the physico-chemical treatments of remediation; consequently, the development of natural techniques for the elimination of pollutants has been encoraged. Bioremediation can be carried out in various forms: natural attenuation, biostimulation, and bioaugmentation.

4.2 Different forms of bioremediation

4.2.1 Natural attenuation

The natural attenuation of pollution refers to the processes contributing to the reduction of pollution in a specific site. It consists on the involvement of the indigenous bacteria present in the environment without any intervention. This approach is passive, thus the process of biological degradation depends only on the natural conditions of the site. A significant reduction of pollution can be attained by this approach but it takes long periods of time. This approach was evaluated by Margesin & Schinner (2001) in a crude oil polluted site. The evaluation of the natural attenuation of polluted sites is an essential task. It aims to predict the fate of the pollution source in the site that allows planning the actions to be undertaken. Even if this technique of decontamination does not require direct human intervention, it is however necessary to intervene in order to eliminate or neutralize the source of pollution and to supervise permanently the site until the end of treatment (Mulligan et al., 2004). This type of bioremediation is not expensive because it does not require additional technical intervention but it requires long time for the treatment to be effective. For instance, Serrano et al. (2007) demonstrated that this process, carried out as a pilot of bioremediation, allowed the restoration of a ground polluted with 2700 hydrocarbon ppm in 200 days. Biodegradation by natural attenuation, cannot however be allotted only to the

microorganism action. In fact, abiotic processes, such as evaporation, dissolution, dispersion, emulsification, adsorption, and photo-oxidation, can also contribute to the natural attenuation of the pollution.

4.2.2 Biostimulation

Biostimulation consists in modifying the natural conditions of a site to increase the rates of biological degradation. Indeed, the intrinsic capacities of an environment to be able to carry out natural attenuation are correlated to several parameters. The first parameter is the biodisponibility of the pollutant which can be affected by the pollutant affinity to the mineral and organic fractions of the environmental matrices. Biodisponibility of the pollutant can be influenced by the organic matter concentratin, the pH, the mineralogy, the temperature, and the type of pollutant.

The second parameter is the oxidation-reduction environment of the ecosystem. Thus, the availability as acceptors or donors of electrons can influence the microbial activities. The biodisponibility of the nutriments (nitrogen and phosphorus) and the microbial associations in the ecosystem can also control the natural attenuation (Röling & Van Verseveld, 2002). Biostimulation improves biodegradation by modifying the environmental conditions through the addition of surfactants, nutrients, water, and chemical species acting as donors or acceptors of electrons. Perfumo et al. (2007) studied the effect of temperature and the addition of nitrogen, phosphate, inorganic potassium, and surfactant on the bioremediation of a ground polluted by hexadecanes. After 40 days of treatment, the grounds treated by the addition of nitrogen, phosphorus and inorganic potassium showed 10% of increase in the degradation compared with the grounds subjected to only natural attenuation. In the same way, Margesin & Schinner (1997) showed the effectiveness of biostimulation in the biodegradation of oil (diesel). In 1991, Hinchee et al. carried out the biostimulation of natural flora in a polluted ground by pumping oxygen. During *in situ* bioremediation, the control and intervention on pH, oxygen addition, and agitation is difficul to apply which makes *in situ* bioremediation expensive. The addition of nutriments is generally adopted. Bragg et al. (1994) carried out a biostimulation by fertilization of the marine environment to stimulate the natural biomass in areas of Alaska following the accident of the Exxon Valdez. Biostimulation of marine environment can be carried out by:

i. Bioventilation: introduction of air within the contaminated zone,
ii. Biosparging: injection of air under pressure,
iii. Pumping and treatment: pumping and purification of the water contaminated by the oil spill,
iv. Addition of small quantity of mineral nutrients to the indigenous microflora. However, this method presents the disadvantage of being no selective and stimulates all types of microorganism present in the site.

4.2.3 Bioaugmentation

This method is based on the addition of *in vitro* cultivated inoculum in the polluted site. The inoculum contains one or more adapted microorganisms, with tested capacities for the degradation of pollutants. Indeed, sometimes, the endogenous microbial populations of polluted ecosystem do not present the metabolic tools to carry on the complete degradation

of the pollutants. Thus, bio-augmentation makes possible to overcome this deficiency by incorporating microorganisms with adapted metabolisms on the level of the contaminated ecosystems. (El Fantroussi & Agathos, 2005). Bioaugmentation can be done by the addition of a stock alone or a group of stocks "consortia". Da Silva & Alvarez (2004) showed an improvement of anaerobic degradation of a mixture composed of Benzene/Toluene/Ethylbenzene/Xylene (BTEX) and ethanol, contained in a polluted aquifer, following the contribution of methanogene consortia. However, multiple factors, especially biotic (predation, competition), can lessen the effectiveness of such techniques while leading to the decline of the introduced populations (Van Veen et al., 1997). In order to mitigate these ecological constraints, physical protection systems of the sown microorganisms, such encapsulation in gellan gum, can be used (Moslemy et al., 2002). Genetic engineering can participate to solve the stability problems of the exogenous stocks. In this context, endogenous microorganisms can be genetically modified in their degradation genes necessary to the improvement of their purifying capacities. Thus, Watanabe et al. (2002) demosntrated an improvement of the decomposition of phenol after the addition of an endogenous microflora genetically modified by the introduction of a gene coding for a phenol-hydroxylase. Certain studies, moreover, established the possibility of improving the effectiveness of the bioremediation by the directly addition of the genes of interest in the polluted ecosystems. In this case, gene transfers between donor bacteria of catabolic plasmid and the endogenous populations allow the biological degradation of pollutants (Bathe et al., 2005). The addition of microorganisms in the environment is not frequently practiced because it is always difficult to control (Van Veen et al., 1997). It is then significant to improve our knowledge on the fate, the persistence, the activity, and the dispersion of the microorganisms injected in polluted sites. In addition, the study of biotreatability must be carried out in the laboratory before being able to choose the biorestoration operation. Indeed, it is after having checked that i) the pollutants are biodegradable, ii) the microorganisms of the site have the capacity to degrade these compounds, iii) the nature of the medium and the environmental conditions are favourable to the development of the microflora, that bioremedation could be set up. It is thus very interesting to characterize the bacterial communities, such as those degrading oil, because the knowledge of the relationship between the microorganisms and their environment is an essential element to understand an ecosystem, and therefore to consider a strategy of bioremediation.

Bioremediation processes are considered interesting alternatives to the traditional techniques of depollution. Indeed, these processes make possible to carry on the decontamination of environments polluted by decreasing the impacts on the treated ecosystems. In order to improve these processes of depollution, it is however necessary to include/understand the operating mode of these microbial communities with respect to the pollutant. Moreover, once natural depollution is initiated; the continuous monitoring of the microorganisms is necessary to make sure that the "biosystème" does not deviate from its initial function. Due to the microorganism's organization in consortia and their specific needs, they are not easily cultivable and their study by traditional microbiological techniques remains consequently limited. Thus, the identification and follow-up of the bacterial stocks being of interest in bioremediation requires the implementation of molecular techniques (Amann, 1995). Different techniques were used with certain success (Margesin & Schinner, 2001; Romantschuk et al., 2000); however, the effective application of these bioremediation techniques still requires considerable research and optimization.

4.3 Biodegradation of oil hydrocarbons in marine environment

Among the microorganisms, bacteria are qualitatively and quantitatively dominant in hydrocarbon degradation. Indeed their capacity to develop in hydrocarbons is not limited to some microbial species. Multiple studies have demonstrated the properties of hydrocarbonoclastic bacteria (Zobell since 1946; Reisfled et al., 1972; Walker & Colwell, 1974, Bartha & Atlas, 1977). The diversity of the species was also established by studies of numerical taxonomy (Bertrand & Thousand, 1989), which showed that the use of hydrocarbons by *Enterobacteriacea* would be acquired by transfer of plasmids. In addition, the composition and the effectiveness of the microflora will be a function of the oil origin, climatic conditions, and seasonal variations (Walker & Colwell, 1974). The biological degradation of a hydrocarbon mixture is not the sum of the biological degradation of each compound taken individually. Olson et al. (1999) evaluated the individual degradation of the principal chemical families of oil (n-alkanes, iso and cyclo-alkanes, and aromatics) compared with the degradation of a fraction made up (mixture) of these three families. This study demonstrated that the susceptibility to biodegradation seems to arise in the following order: n-alkanes > aromatic > iso and cyclo-alkanes.

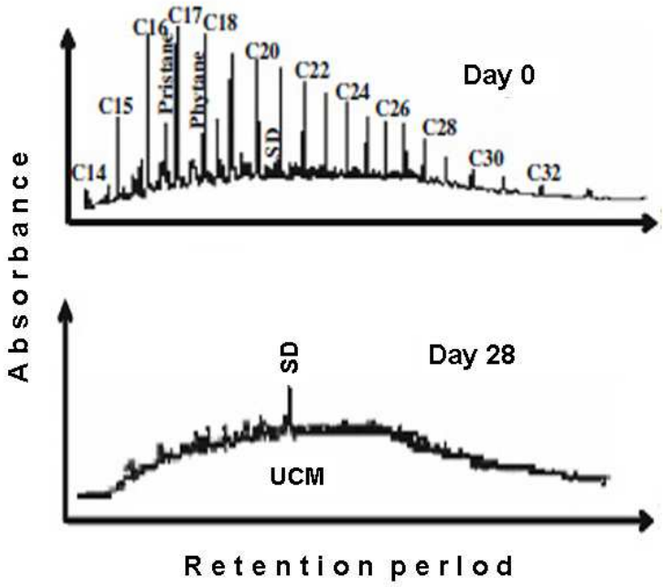

Fig. 4. Chromatogram spectra showing the biodegradation of non aromatic hydrocarbon (NAH) fractions, at different incubation periods (Zrafi-Nouira et al., 2009); UCM: Unresolved complex mixture; SD: standard.

The individual degradation of hydrocarbon compounds has been extensively studied for years. However, few studies have focused on the evaluation of the biodegradation of mixtures of complex products as crude oils. The aliphatic fraction is degraded more quickly than the aromatic fraction. However, the degradation is slower for mixtures of compounds than on the individual compounds. Data concerning the degradation of oil result often from

observations carried out on polluted sites, i.e. open ecosystems, in which a complete assessment of the biodegradation process is not possible. Several studies concerning petroleum biodegradation, on site or in the laboratory, were carried out by Salanitro, (2001); who proved that the rates of degradation were often over-estimated, thus stressing the importance to work in a control environment (laboratory) to effectively carry out the assessment of the carbon degradation tests. Research has shown that the degradation of complex mixtures as oil depends on various types of bacteria whose catabolic capacities are complementary can thus cooperate in the degradation. Richard & Vogel (1999) isolated a consortium from the ground containing seven different bacteria (five *Pseudomonas*, an *Achromobacter,* and another bacterium that was not identified) of which only three were able to use hydrocarbons as growing substrate. It seems that the rest of the bacteria present in the consortium used the metabolic intermediaries as growing substrate. Head et al., (2006) observed that in marine environment following an oil spill, there is a correlation between hydrocarbon composition and bacteria dynamics. Bacteria take a significant share in natural decontamination following an oil discharge because of their capacities to quickly adapt to changing conditions. Figure 4 shows chromatograms showing non aromatic hydrocarbon (NAH) fractions, at different incubation periods during crude oil (Zarzatine) degradation by microflora in polluted seawater from Tunisia.

Table 1 summarizes the biodegradation of different carbon sources and briefly describes the microorganisms involved in the process.

Carbon Sources	Incubation period	Biodegradation %	Microorganisms description	Reference
Zarzatine Crude oil (ZCO)	28 days		Adapted microflora from polluted seawater adjacent to oil refinery (Tunisia)	Zrafi-Nouira et al.(2009)
Saturated fraction		92.6		
Aromatic fraction		68.7		
Oil refinery tank bottom sludge (OTBS)	10 days		Adapted microbial consortia from polluted soil	Gallego et al. (2007)
n-alkanes		100		
Branched alkanes		44		
cyclo-alkanes		85		
aromatics		31-55		
Crude oil	10 weeks		Microbial consortium from seawater near a oil refinery (UK)	McKew et al. (2007)
n-alkanes (C10-C18)		99		
n-alkanes (C20-C32)+Pr		41-84		
PAHs		32-88		
Crude oil	7 days		Seawater adjacent to oil refinery (Tunisia)	Ozaki and Fujita (2006)
Saturated fraction		56		
Aromatic fraction		46		
Saturated + aromatics		20		
Crude petroleum	200 days		Eubacterial from soil contaminated with wood treatment plant (near Barcelone)	Vinas et al. (2005)
TPH		72-79		
PAHs		83-87		

Pr: Pristane; TPH: total petroleum hydrocarbons; PAHs : Polycyclic aromatic hydrocarbon

Table 1. Biodegradation of crude oil compounds.

5. Place of metagenomics in petroleum bioremediation

5.1 From culture-dependent approaches to culture-independent approaches

Investigations of microbial species that are present in petroleum polluted environments and during petroleum spill bioremediation have been traditionally conducted using samples to grow bacterial cultures in the laboratory. However, laboratory growth medium does not reproduce the actual diversity of the complex system (polluted environment). The current culture-dependent methods used can only account for a small subset of the total microbial diversity. This causes the underestimation of the microbial communities present in petroleum polluted environments. The challenge of isolating even a small fraction of these organisms seems overwhelming (Tyson & Banfield, 2005). Traditional microbiological methods of cultivation recover less than 1% of the total bacterial species present in the polluted site sample, and the cultivable portion of bacteria is not representative of the total phylogenetic diversity. Indeed, most of the cultivated microorganisms are those that grow quickly and are capable to growth in nutrient-rich media (Leadbetter, 2003) and those that are dominant and key players in the environmental system.

Thus, an improvement of cultivation methods has recently been addressed and non-traditional culture methods have been developed (Green & Keller, 2006; Tyson & Banfield, 2005). These methods are based on modified traditional approaches to isolate previously uncultured and phylogenetically distinct microorganisms that grow in diluted nutrient media or simulated natural environments (Green & Keller, 2006; Tyson & Banfield, 2005). Non-traditional approaches include single cell manipulation techniques such as optical tweezers and laser microdissection, targeted isolation using 16S rRNA-direct probes, and the development of high-throughput methods to grow encapsulated single cells (Tyson & Banfield, 2005). Despite these advances, cultivation attempts of petroleum degrading-bacteria and their isolation from complex polluted site samples fail. Genetic methods can be used to overcome the difficulties associated with the laboratory cultivation of petroleum-degrading-bacteria, which depend upon environmental conditions, unsuspected growth factors, and multiples relationships within a complex community. The use of genetic techniques to detect, identify, and quantify bacteria has largely replaced microbial growth tests. These modern biotechnology methods have been recently employed in petroleum polluted environments. Genetic techniques will be the methods of choice for monitoring bioremediation bacteria players in the future. Initial efforts to introduce the use of genetic techniques for monitoring petroleum-degrading-bacteria in environmental samples have involved a type of biotechnology called metagenomic. There are molecular techniques available that can be used to study genomic material obtained directly from the environment, instead of cultured samples, which provides the opportunity to extract sequence data from microbial communities as they exist in nature. Extraction of genome sequences from metagenomic data is crucial for reconstructing the metabolism of microbial communities that cannot be mimicked in the laboratory.

Crude oil metagenomics could be used to examine the bacteria dynamics during petroleum biodegradation and *in situ* bioremediation in hydrocarbon polluted sites.

The comprehension of petroleum polluted environments and the identification of the microorganisms playing key roles during biological degradation remain uncertain so that the optimal development of bioremediation strategies is still unknown. The advantages of

these molecular techniques are that they allow accessing the microbial diversity of complex systems (Theron & Cloete, 2000) and allow supervising and evaluating the bioremediation process (Bachoon et al., 2001; Brockman, 1995; Widada et al., 2002). Most of these studies using molecular techniches were based on the construction of 16S rRNA clone libraries and subsequent sequencing of individual 16S rRNA clones. The resulting nucleotide sequences were then taxonomically and phylogenetically classified to deduce the structure of the underlying community.

5.2 Molecular tools to monitor bacterial composition in petroleum polluted sites

Assessing environmental microbial community structures through molecular techniques requires a satisfactory sampling strategy that takes into account the high microbial diversity and the heterogeneous distribution of microorganisms in polluted sites. So that, microbial ecologists have invested substantial effort in optimizing DNA and RNA extraction procedures from various environmental samples (water, sediments, soil and sludge).

The key molecular player in microbial classification has been the RNA component of the small subunit of ribosomes (SSU rRNA, or 16S/18S rRNA), which Carl Woese in the early 1970s considered to be a convenient and reliable « universal molecular chronometer ». These nucleic acids contain preserved regions allowing an easy isolation and variable regions allowing the differentiation of taxonomic units. These biological molecules are the targets of choice for molecular ecology studies (Olsen et al., 1986).

The screening of metagenomic libraries has traditionally followed two paths: sequence-based and function-based screening. Some of the metagenomic libraries have been screened by hybridization or by PCR to detect genes with homology to known genes. The analysis of genome sequence data that has been recovered from the environment is motivated by many objectives, which include the establishment of gene inventories and natural product discovery (Handelsman, 2004). Genomics can resolve the phyogenetic composition and metabolic potential of communities during bioremediation processes and establish how functions are played among populations, reveal the dynamics of population diversity in response to changes in hydrocarbon changes during biodegradation of petroleum compounds. The evaluation of bioremediation can be studied by molecular fingerprinting techniques applied to degradation genes. The polymorphism of genes considered is detected by enzymatic restriction and examination of the profiles of restriction after genic amplification. Watanabe et al. (1998) used temperature gradient gel electrophoresis (TGGE) of gene coding a phenol hydroxylase (LmPH) and gene coding 16S rRNA in order to detect and to characterize the prevalent bacteria degrading phenol in activated sludge of a station of purification. MacNaughton et al. (1999) used denaturing gradient gel electrophoresis DGGE to identify the populations responsible for decontamination and to evaluate two techniques of bioremediation after oil spill (biostimulation and bioaugmentation). Two sequences affiliated to *Flexibacter-Cytophaga-Bacteroides* were detected in the biostimulated samples but the participation of these microorganisms in the hydrocarbon degradation was not proven.

The combination of several methods offers interesting information in microbial diversity of the studied ecosystems. Watts et al. (2001) recently compared three methods using 16S rDNA: ARDRA (amplified ribosomal DNA restriction analysis), T-RFLP (Terminal-

restriction fragment length polymorphism), and DGGE that were applied to study the communities involved in the degradation of chlorinated compounds. They conclude that the three methods render different results, namely the relative frequency of the clones for ARDRA, intensity of the bands for the DGGE (resolution of freezing) and the peaks height for the T-RFLP. However, the use of these three different methods seems to allow an appropriate analysis of the studied communities and the information obtained from the different methods employed was complementary.

It is also interesting to couple the analysis of microbial diversity with a degradation activity study. Kanaly et al. (2000) used the DGGE technicque to access the dynamics of a bacterial consortium during the degradation of benzo[α]pyrene. They confirmed the presence, in their consortium, of microorganisms known to degrade the aromatic compounds, such as *Sphingomonas paucimobilis EPA505*, *Mycobacterium str.PYR-1,* and *Alcaligenes denitrificans WW1*. In addition, it is interesting in bioremediation evaluation to detect the functional degradation genes. The expression of these genes can be detected based on ARNm analysis (Van Hamme et al., 2003). The extraction of ARNm followed by RT-PCR (Reverse transcription polymerase chain reaction) coupled to an analysis of 16S rDNA makes possible to get evidence of the active microorganisms in a microflore. These methods offer an interesting alternative to detect the activities of non- cultivated bacteria.

In fact, certain degradation pathways are well characterized and some degradation genes are well known, which allowed the development of a large variety of specific primers. Currently, the detection of the *alk*B gene, implied in the metabolism of alkanes, and others genes implied in the metabolism of aromatic hydrocarbons is well documented (Whyte et al., 1997; Whyte et al., 1998; Churchill et al., 1999; Smits et al., 1999; Widada et al., 2002). Margesin et al. (2003) showed that the genotypes of alkB of *P. putida* are more frequently found in high-altitude polluted grounds than in the not polluted grounds. Wilson et al. (1999) developed a method to isolate and characterize the ARNm of microorganisms degrading naphthalene in a hydrocarbon contaminated aquifer. Recently Debruyn et al. (2011) examined the homologous genomic regions in four PAH-degrading Mycobacterium (strains JLS, KMS, MCS, and M. gilvum PYR-GCK) isolated from two PAH-contaminated sediments. These isolates had nidA (and some, nidA3) genes that were homologous to Mycobacterial ARHDO (aromatic ring hydroxylated dioxygenase) genes, suggesting that horizontal gene transfer events had occurred. Militon et al. (2007) proposed a probe design algorithm that is able to select microarray probes targeting SSU rRNA at any phylogenetic level. Application of the combined array strategy may help identifying unknown bacterial species. Neufeld et al. (2006) used microarray technology to characterize and compare hexachlorocyclohexane (HCH) contaminated soils from Spain and their results highlighted the power of habitat-specific microarrays for comparing complex microbial communities. Gomes et al. (2010) used the combination of culture enrichments and molecular tools to identify bacterial guilds, plasmids, and functional genes potentially important in the process of petroleum hydrocarbon decontamination in mangrove microniches (rhizospheres and bulk sediment), and to recover degrading consortia for future use in remediation strategies. Fingerprinting could be obtained with proteomics and metabolomics approaches but results are difficult to relate to a precise identification at the phylogenetic level. Databases must be constructed and related genomics, proteomics, and metabolomics information to obtain easily interpretable results for microorganism identification. In fact, shotgun sequencing approaches have been used to reconstruct genome fragments and near complete genomes

from uncultivated species and strain from natural consortia (Tyson & Banfield, 2005). Sequence data from uncultivated consortia are more useful for metabolic profiling if the genome fragment can be assigned to an organism type. Sequence similarity to previously characterized organisms and the presence of phylogenetically informative gens would assist genomes reconstruction. Wang et al. (2011) generated a complete *Methanococcus maripaludis* genome from metagenomic data derived from a thermophilic subsurface oil reservoir. Comparison of the genome from the thermophilic, subsurface environment with the genome of the species type provided insight into the adaptation of a methanogenic genome to an oil reservoir environment. Thus a robust database of genomic information from cultivated microorganisms can greatly enhance the ability to link genome fragments to a particular organism (Figure 5). Currently, both the cost of sequencing and the challenges that are associated with the management of vast datasets precludes comprehensive genomic studies of highly complex communities. It would be optimal if the amount of sequencing is appropriate with the diversity of the sample to be analyzed.

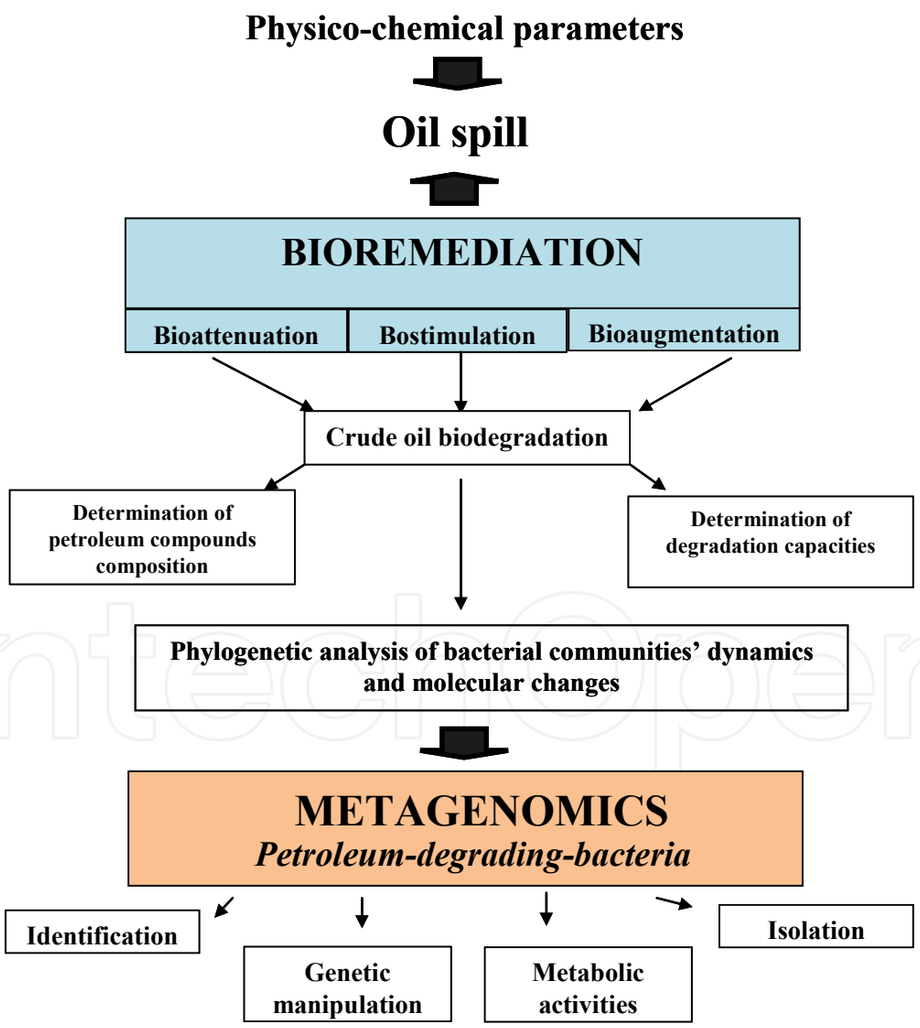

Fig. 5. Schematic representation of the bioremediation process of an oil spill and its possible improvement by metagenomics.

5.3 An overview of phylogenetic analysis reports in characterising bacteria composition in polluted environments

The analysis of bacterial diversity in different polluted environments helped to discover the black box of adapted consortia dynamics in response to the presence of petroleum compounds. Phylogenetic analysis of different consortia originated from petroleum polluted environment shows that the abundant phylotypes distributed within the group of *Proteobacteria* are essentially *alph-, beta-,* and *gamma-proteobacteria*. The predominance of *Proteobacteria* in polluted hydrocarbons environments has been largely documented (seawater, ground, ice, estuary, and river). Thus the prevalence of *Proteobacteria* in polluted seawater is well known (Brakstad & Lødeng, 2004; Brakstad et al., 2004; Kasai et al., 2005; Marc et al, 2005). Using hydrocarbon polluted sand, Mac-Naughton et al. (1999) showed the predominance of the *Alphaproteobacteria* compared to the *Gammaproteobacteria*. They also reported that *Gammaproteobacteria* are detected after 8 weeks following contamination by oil. Popp et al. (2006) concluded that the predominance of *Gammaproteobacteria* can be correlated to high levels of contamination. McKew et al. (2007) reported that the indigenous microflora from an estuary in Brittany (France) adapted to crude oil degradation were dominated by *Thalassolitucus Oleivorans* and were composed of various other phylum such as *Oceanospirillum, Roseobacter*, and *Arcobacter.* In grounds contaminated by crude oil in the north of Canada, Juck et al. (2000) showed that the bacterial population was composed mainly by *Nocardioides, Arthrobater,* and *Xanthomonas*. In Germany (the North Sea) Brakstad & Lødeng (2004) reported that the indigenous microflora of polluted environment was characterized by the presence of *Sphingobacteria Flavobacteria, Pseudoalteromonas, Alteromonas, Vibrio,* and *Roseobacter*. The bacterial population of a polluted ground zone of Shizuoka, in Japan (Kasai et al., 2005) mainly contained *Variovorax, Acidovorax, Burkholderia, Thiobacillus, Alcaligens,* and other microorganisms. In the clone library obtained from asphalts of the tar wells in California, the prevalent bacteria were affiliated to *Chromatiales, Xanthomonadaceae*, *Pseudomonadaceae,* and *Rhodobacteraceae* (Kim & Crowley, 2007). *Moraxellaceae* within the *Acinetobacter* are often present in polluted sites with shown capacities to degrade linear alkanes in C16 (Bogan et al., 2003). The presence of *the Xanthomonas*, *Stenotrophomonas,* and *Hydrocarboniphaga* has been reported (Popp et al., 2006). Similarly, the abundance of other dominant groups in polluted environments has also been reported; in fact, *Actinobacteria*, *Firmicutes,* and *Bacteroidetes* were described to be also dominant (Chang et al., 2000; Popp et al., 2006). Recently, Dos Santos et al. (2011) studied the effect of the oil spill on the existing microbial community using direct pyrosequencing, which confirmed that the phylum *Proteobacteria*, in particular the classes *Gammaproteobacteria* and *Deltaproteobacteria*, were prevalent before and after the simulated oil spill. The authors claim that the order *Chromatiales* and the genus *Haliea*, and three other genera, *Marinobacterium*, *Marinobacter,* and *Cycloclasticus* increased, which make them possible targets for the biomonitoring of the impact of oil in mangrove settings.

The difference in bacteria group dominance in petroleum polluted environment indicates that indigenous population is specific to this setting. These differences can be allotted to the environmental conditions (temperature, pH, salinity, O_2, etc.) and the nature of the petroleum compounds present in the site (crude oil, refined product, asphalt, gasoil, etc).

The dominant described taxa represent specific bacteria that are strongly resistant to the presence of petroleum hydrocarbon. General literature (Zrafi-Nouira et al., 2009; Head et al.,

2006; Popp et al., 2006) describes a range of phylogenetic bacteria groups in which the most abundant taxa are closely related to: *Pseudoaminobacter, Alcaligenes Nitrate redactor* and *Halomonas*, *Alcanivorax, Pseudomonas , Mesorizobuim , Shewanella,* and *Marinobacter*.

Indeed various indigenous total colony counts were worldwide described, and each microflora was specific to its environment. These studies described various microbial communities which can live on the complex mixtures of oil hydrocarbons. Nevertheless, little is known on the changes of the consortia after a specific enrichment by crude oil (Zrafi-Nouira et al., 2009).

5.4 An overview of bacterial dynamics during *in vitro* biodegradation and *in situ* bioremediation

There is a general interest to study the microflora diversity that is able to degrade crude oils (Abed et al., 2002; MacNaughton et al., 1999; Orphan et al., 2000).

The phylogenetic analyses carried out in various stages of the biodegradation incubation during crude oil degradation showed differences in the chemical composition of the oil and the microbial composition of the communities (Table 2). Brakstad & Lødeng (2004) indicated that the reduction of microbial diversity is observed from day 7 to day 21 during the degradation of crude oil. Indeed, the presence of oil reduces clearly bacteria diversity (Röling et al., 2002). During biodegradation bacterial divisions persist dominant but with a variation of their proportions and their compositions.

Library	Affiliation group	Affiliation sub-group	Number of clones	Number of OTUs	% of OTUs	% of cultivated OTUs	% of uncultivated OTUs	% of novel OTUs
Adapted microflora	Proteobacteria	Alpha	27	8	36.4	87.7	12.5	0
		Beta	36	3	13.6	66.7	0	33.3
		Gamma	14	6	27.3	83.3	0	16.7
	Firmicute	Clostridia	13	3	13.6	33.3	0	66.7
	Actinobacteria	Actinobacteria	3	1	4.55	0	0	100
	Bacteroidetes	Flavobacteria	1	1	4.55	100	0	1
Day 7	Proteobacteria	Alpha	43	8	29.6	50	0	50
		Beta	3	1	3.7	100	0	0
		Gamma	49	16	59.3	62.5	0	37.5
	Firmicute	Clostridia	2	1	3.7	100	0	0
	Bacteroidetes	Flavobacteria	2	1	3.7	100	0	0
Day 14	Proteobacteria	Alpha	68	7	58.4	42.8	0	57.2
		Beta	5	1	8.3	0	100	0
		Gamma	13	3	25	0	33.3	66.7
	Actinobacteria	Actinobacteria	1	1	8.3	0	0	100
Day 21	Proteobacteria	Alpha	8	2	22.2	100	0	0
		Beta	2	1	11.1	0	100	0
		Gamma	45	5	55.6	80	0	20
Day 28	Actinobacteria	Actinobacteria	1	1	11.1	0	100	0

[a]: The number of OTUs was calculated with a threshold value of 97% 16S rRNA sequence similarity

Table 2. Distribution of clone sequences and OTUs analyzed during *in vitro* crude oil biodegradation using an adapted bacterial microflora from polluted seawater (Zrafi-Nouira et al., 2009).

Several studies suggest a balance between Alpha- and Gamma-Proteobacteria, which depend on the nature, level, and composition of the oil sources. In our previous study (Zrafi-Nouira et al., 2009); we noticed that after day 7 of crude oil degradation the microbial flora from polluted seawater was significantly diversified. We also noted the appearance of a novel Operational Taxonomic Unit (OTUs) with less than 97% of similarity with the nearest parent in the GenBank database. This study made possible to identify bacterial phylotypes potentially implied in the processes of decomposition of aliphatic and aromatic hydrocarbons during the degradation of crude oil.

We identified a potential implication of *Gamma-Proteobacteria*: *Alcanivorax, Halomonas, Enterobacter, and of Uncultred bacteruim*. We noticed a phenomenon named "gamma-shift", which consists of a deviation of diversity towards the γ-Proteobacteria during an increase in the nutrients of the contamination source (Popp et al., 2006). According to Evans et al. (2004), this tendency could be caused by a high number of bacteria degrading hydrocarbons belonging to this class (γ-Proteobacteria*)*. The abundance of bacteria from the *Alpha-Proteobacteria,* primarily of the *Pseudaminobacter* also seems to suggest their adaptation to the presence of hydrocarbons. These bacteria are even found during the first phases of degradation. Indeed, the detected species *Pseudaminobacter sp. W11-4* A was described as pyrene degrading bacteria. However, after the 28 day of degradation, only the *Actinobacteria* were present in the consortium. We hypothesized that at this stage the prevalent species are implied in the decomposition of recalcitrant aromatic and cyclic hydrocarbons with the possibility of the anaerobic decomposition of the residual hydrocarbons. Similarly, another study showed the association of *Actinobacteria* in the anaerobic decomposition of hydrocarbons oil (Rehmann et al., 2001; Pinda-flora et al., 2004). *In vitro* studies of the crude oil degradation process provides insights on the composition of the bacterial communities and their evolution during degradation. It is thus essential to be able to identify the purifying bacteria composing this microflora and to understand their adaptive mechanisms in the presence of petroleum. Thus, information obtained in this regard could guide the installation of processes stimulating the activity of the bacteria implied in the degradation of the pollutant or allowing their massive establishment in an environment to be treated *in situ*. The bacterial composition is then of primary importance for the comprehension of the process of *in situ* bioremediation after oil spills. The study of bacterial dynamics and the diversity during the natural attenuation, biostimulation or bioaugmentation of the oil discharge can help identifying bacterial phylotypes potentially implied in the biodegradation processes. Indeed during bioremediation, we can observe an evolution of the bacterial communities which seems to be correlated with the reduction of oil charge in the sites. This could be due to the decomposition of hydrocarbons by specialized microorganisms that generates the intermediate compounds, which produce new pressures of selection on the microbial communities involving their evolution. The adapted bacteria persist and even grow and those not having the adaptive mechanisms decline because the conditions in this environment do not promote their development. Observations conducted during bioremediation indicated that *Proteobacteria* decreased while *Actinobacteria* increased. Although bacterial communities show evolution during bioremediation, the presence of some phylotypes seems to be constant, which is named bacterial "core-set". This "core-set" is composed of phylotypes belonging primarily to *Actinobacteria α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, and Flavobacteria*.

Biotreatability of contaminated sites must be carried out first in the laboratory setting to gain knowledge on the proper biorestoration operation; nevertheless the *in situ* study is needed to access real information of the bioremediation process in nature. *In situ* characterization of bacterial communities degrading oil provides information on the relationship between microorganisms and the environment. In addition, the composition and the effectiveness of the microflora will be a function of the origin of oil and the environmental conditions (Harayama et al., 1999). Several studies concerning biodegradation of petroleum products, on site or at laboratory scale, were carried out (Salanitro, 2001). Some studies have proved that the rates of degradation were often over-estimated in laboratory evaluations. An explanation for this might be the fact that each medium has a potential of degradation and a specific indigenous microflora, so that similar contamination does not cause the development of similar microbial communities (Bundy et al., 2002). In consequence, high concentration of pollutants decreases the density of the microbial population, whereas a lower content of pollutants enriches the microflora able to degrade hydrocarbons (Long et al., 1995). According to Smit et al. (2001), the distribution of bacteria would be rather correlated with the availability of nutrients. Indeed, availability of the substrates would select species supporting a fast and massive division, such *Proteobacteria* whereas a limited availability of nutriments would support the species having slower but more effective division like the *Acidobacterium* and *Actinobacteria.* These two theories are however not conflicting if we take into account that in marine environment the presence of unstable niches from the nutritive point of view will support microorganisms with fast adaptation.

In addition, the interactions between various species (phenomena of co-operation), with variable generation times from one species to another allows the relative abundance of each species within the population (a less abundant population can replace abundant population but less adapted to the selected culture medium) that can act in the selection of the microflores on site (Amann et al., 1995; Ward et al., 1997).

Comparison between *in vitro* and *in situ*, based on the bacterial composition analysis changes and its dynamics during bioremediation, is valid for a better comprehension of these processes and their applications at various levels (decontamination of the polluted sites, industrial and domestic effluent, and waste processing). There is a difference in the fine composition of the bacterial phylotypes. The microflora acting on site has a wealth of bacterial diversity, whereas the microflora obtained during various incubation periods at laboratory scale are less diverse and more specific. This is the result of multiple factors: the effects of the conditions of the sites, the interference of others organic compounds present on site, the interference of the microorganisms present in other compartments of the environment (sediments and rocks). For the microflora acting *in vitro,* multiple factors can intervene on the bacterial composition. The major one is the effect of acclimation to crude oil. Indeed, acclimation supports a specific selection to the type of oil used. As well as the effect of the selection that is imposed by the contribution of continuous pollution sources on the indigenous microflora. Under laboratory conditions, the adapted microflora is subjected to the effect of the metabolites and their accumulation in a closed microcosm that can be toxic and limit their growth. In addition to these differences within the phylotypes, a bacterial "core-set" was highlighted in terms of bacterial groups. Indeed, the bacterial inventories (Zrafi-Nouira et al., 2009) showed the prevalence of *Proteobacteria* in the majority of the cases with a swinging between *Gamma* and *Alpha Proteobacteria* in terms of abundance

and diversity according to the nature and the degree of the contamination. A proliferation of *Gamma proteobacteria* phenomenon called "Gamma-shift" could be related to an enrichment of polluting compounds. During *in vitro* degradation and *in situ* bioremediation the proliferation of *Gamma proteobacteria* was observed in the early phases of degradation, whereas an exclusive abundance of *Actinobacteria* was observed in the late phases of bioremediation. We suggested an implication of these *Actinobacteria* in the decomposition of recalcitrant branched hydrocarbons or aromatics present at the end of the degradation process. The adaptation of the bacterial populations following petroleum pollution can highlight the degrading capacities of certain microorganisms. Thus, in order to identify the bacteria having these abilities, comparison of population dynamics in different polluted matrices is useful to monitor active spices.

6. Conclusions

In nature and especially in marine environments crude oil is considered to be an important source of pollution. After oil spills accidents, petroleum products are subjected to environmental changes resulting in their degradation. However, human intervention through the application of bioremediation technology is necessary to accelerate the bioremediation process. Advances in bioremediation technologies are based in the improvement of our comprehension of the composition of the microflora present in the polluted site and the activity of the microorganisms implied in petroleum degradation, which help to select bioremediation strategies for polluted sites. It is thus necessary to develop a multi-field approach taking into account all the parameters intervening in the natural bioremediation process. The use of methods based on the molecular approach could be helpful to improve the evaluation of the diversity, identification, isolation, and functional properties of microorganisms involved in the degradation of different petroleum compounds. The molecular analysis of the bacterial composition present in polluted sites represents an essential stage to improve our understanding of the microorganisms involve in the degradation of hydrocarbons. Similarly, metagenomics represent the technology of choice to enhance the application of bioremediation in the future. The key point of this review is that nature seems to offer a diversity of bacteria capable of degrading and neutralize crude oil pollution. It is up to us to optimize the application of what nature provides.

7. References

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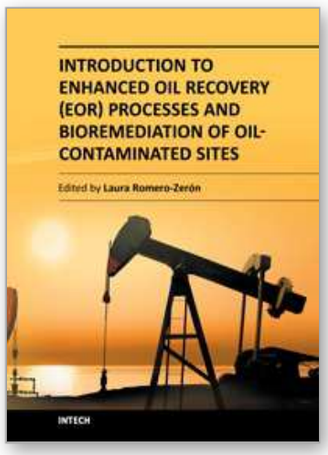

Introduction to Enhanced Oil Recovery (EOR) Processes and Bioremediation of Oil-Contaminated Sites

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This book offers practical concepts of EOR processes and summarizes the fundamentals of bioremediation of oil-contaminated sites. The first section presents a simplified description of EOR processes to boost the recovery of oil or to displace and produce the significant amounts of oil left behind in the reservoir during or after the course of any primary and secondary recovery process; it highlights the emerging EOR technological trends and the areas that need research and development; while the second section focuses on the use of biotechnology to remediate the inevitable environmental footprint of crude oil production; such is the case of accidental oil spills in marine, river, and land environments. The readers will gain useful and practical insights in these fields.

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